

# Spleen Tyrosine Kinase Syk Is Necessary for E-Selectin-Induced $\alpha_L\beta_2$ Integrin-Mediated Rolling on Intercellular Adhesion Molecule-1

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## SUMMARY

Engagement of neutrophils by E-selectin results in integrin activation. Here, we investigated primary mouse neutrophils in whole blood by using intravital microscopy and autoperfused flow chambers. Slow rolling on E-selectin coimmobilized with intercellular adhesion molecule-1 (ICAM-1) required P-selectin glycoprotein ligand (PSGL)-1, was dependent on  $\alpha_L\beta_2$  integrin (LFA-1), and required continuous E-selectin engagement. Slow rolling was abolished by pharmacological blockade of spleen tyrosine kinase (Syk) and was absent in *Syk*<sup>-/-</sup> bone-marrow chimeric mice. Treatment with tumor necrosis factor- $\alpha$  lowered rolling velocity further and induced CXC chemokine ligand-1 (CXCL1) and CXC chemokine receptor-2 (CXCR2)-dependent leukocyte arrest on E-selectin and ICAM-1. Arrest but not rolling was blocked by an allosteric inhibitor of LFA-1 activation. Neutrophil recruitment in a thioglycollate-induced peritonitis model was almost completely inhibited in *Selp1g*<sup>-/-</sup> mice or *Syk*<sup>-/-</sup> bone-marrow chimeras treated with pertussis toxin. This identifies a second neutrophil-activation pathway that is as important as activation through G protein-coupled receptors (GPCRs).

## INTRODUCTION

E-selectin is expressed on inflamed endothelial cells and engages P-selectin glycoprotein ligand (PSGL)-1 (Xia et al., 2002), CD44 (Katayama et al., 2005), macrophage antigen (Mac)-1 ( $\alpha_M\beta_2$ ) (Crutchfield et al., 2000), and other ligands. E-selectin is known to mediate slow leukocyte rolling in vivo (Kunkel and Ley, 1996). Slow rolling requires the presence and function of  $\beta_2$ -integrins (Jung et al., 1998). Rolling of isolated human neutrophils on E-selectin

induces p38 MAPK-dependent adhesion to inflamed endothelial cells or cells transfected with E-selectin and intercellular adhesion molecule (ICAM)-1 (Simon et al., 2000). However, when neutrophils are rolled over immobilized E-selectin and ICAM-1 in their physiological whole-blood environment, rolling is greatly enhanced compared to immobilized E-selectin alone, but no firm adhesion is seen (Chesnutt et al., 2006).

Neutrophils can also activate their  $\beta_2$ -integrins and arrest in response to immobilized chemokines (Rainger et al., 1997). The most relevant chemokine receptor responsible for arrest of mouse neutrophils under conditions of inflammation in vivo was recently identified as CXC chemokine receptor-2 (CXCR2) (Smith et al., 2004a). In reconstituted systems, immobilized chemokines are sufficient to trigger arrest of model leukocytes within less than one second (Campbell et al., 1998; Grabovsky et al., 2000).

During inflammation, endothelial cells express both CXC chemokine ligand-1 (CXCL1, formerly known as keratinocyte-derived chemokine, KC), an effective arrest chemokine that activates CXCR2 (Smith et al., 2005), and E-selectin. The E-selectin-dependent and chemokine-dependent recruitment pathways are partially redundant, because blocking either one alone blocks neutrophil recruitment to the inflamed peritoneal cavity by approximately 50%, but blocking both almost completely eliminates neutrophil recruitment (Smith et al., 2004a). In vivo, neutrophils integrate signals from chemokine and E-selectin engagement to eventually become adherent (Kunkel et al., 2000). The E-selectin ligand on neutrophils responsible for integrin activation and subsequent slow rolling is unknown.

PSGL-1 is a ligand for all selectins and binds L- and P-selectin through its NH<sub>2</sub>-terminal sialyl Lewis x (sLe<sup>x</sup>)-containing O-glycan and a nearby tyrosine sulfate residue (McEver, 2002; McEver and Cummings, 1997). E-selectin binding to PSGL-1 requires sialylated and fucosylated O-glycans but not tyrosine sulfatation (McEver and Cummings, 1997). PSGL-1-deficient mice show a pronounced impairment of P-selectin-dependent neutrophil rolling, adhesion, and recruitment, whereas E-selectin-dependent rolling and adhesion is only mildly impaired (Xia et al.,

2002; Yang et al., 1999). Binding of P-selectin to PSGL-1 on leukocytes generates signals that are thought to be integrated with those from other activators (Zimmerman et al., 1996): the expression of transcription factors, chemokines, and cytokines is enhanced when leukocytes receive simultaneous signals via PSGL-1 and G protein-coupled receptors (Galt et al., 2001; Lindemann et al., 2005; Weyrich et al., 1995, 1996). Furthermore, engagement of PSGL-1 induces tyrosine phosphorylation and activation of MAP kinases in human neutrophils, as well as activation of  $\beta_2$ -integrin binding to ICAM-1 (Hidari et al., 1997; Simon et al., 2000).

Upon PSGL-1 engagement, the cytoplasmatic tail of PSGL-1 becomes associated with the spleen tyrosine kinase (Syk) (Urzaizqui et al., 2002). Syk, a nonreceptor tyrosine kinase found in hematopoietic cells, has two SH2 domains, which are involved in the binding to the immunoreceptor tyrosine-based activation motifs (ITAM) of the actin-linking proteins moesin and ezrin in the PSGL-1 complex and other surface receptor complexes (Berton et al., 2005). This leads to recruitment and activation of Syk with subsequent phosphorylation of intracellular substrates. Downstream signals from PSGL-1 engagement are incompletely understood (Abbal et al., 2006). Syk is also involved in integrin outside-in signaling (Mocsai et al., 2002; Schymeinsky et al., 2006).

Neutrophils express lymphocyte function antigen (LFA)-1 ( $\alpha_L\beta_2$ ), Mac-1, and small amounts of  $\alpha_x\beta_2$  (gp 150,95) (Parkos, 1997). Both LFA-1 and Mac-1 (Dunne et al., 2002) have been found to contribute to neutrophil adhesion in inflamed microvessels in vivo. LFA-1 undergoes a series of conformational changes resulting in partial and full activation (Laudanna, 2005). Allosteric inhibitors of LFA-1 activation were recently shown to stabilize a partially activated form of LFA-1 (Salas et al., 2004; Shimaoka et al., 2003a) that supports rolling on ICAM-1 but not firm adhesion.

The first suggestion that E-selectin engagement may activate  $\beta_2$ -integrins came from observations that neutrophils that adhered to TNF- $\alpha$ -stimulated human umbilical vein endothelial cells (HUVECs) showed increased rosetting with C3bi-coated erythrocytes, reflecting activated Mac-1 (Lo et al., 1991). This Mac-1 activation was completely prevented when E-selectin was blocked (Lo et al., 1991). The relevance of E-selectin binding for integrin activation was confirmed in a flow-chamber assay with Ficoll-isolated human blood neutrophils on L-cells transfected with E-selectin alone or with ICAM-1 (Simon et al., 2000). Ficoll-isolated neutrophils undergo a number of phenotypic changes during and after the isolation process (Forsyth and Levinsky, 1990; Glasser and Fiederlein, 1990; Kuijpers et al., 1991) and cannot maintain rolling at high wall shear stress levels similar to those in vivo.

We reasoned that it would be worthwhile to revisit E-selectin signaling by using neutrophils in their native whole blood, without any isolation procedure that might alter their activation status and rolling ability. To this end, we employed a recently developed autoperfused flow chamber (Chesnutt et al., 2006; Smith et al., 2004b, 2006),

which allows direct visualization of neutrophil interactions with a molecularly defined substrate. Because E-selectin is not as efficient as P-selectin at inducing leukocyte capture (Smith et al., 2004b), we increased the E-selectin concentration, which resulted in lower rolling velocities than typically seen in vivo, where both E- and P-selectin are expressed. To confirm the physiological relevance of the findings made in the autoperfused flow chamber, we studied neutrophil rolling and adhesion by intravital microscopy and neutrophil recruitment in a thioglycollate-induced model of peritonitis. Our results identify a second neutrophil-activation pathway that is as important as activation through G protein-coupled receptors.

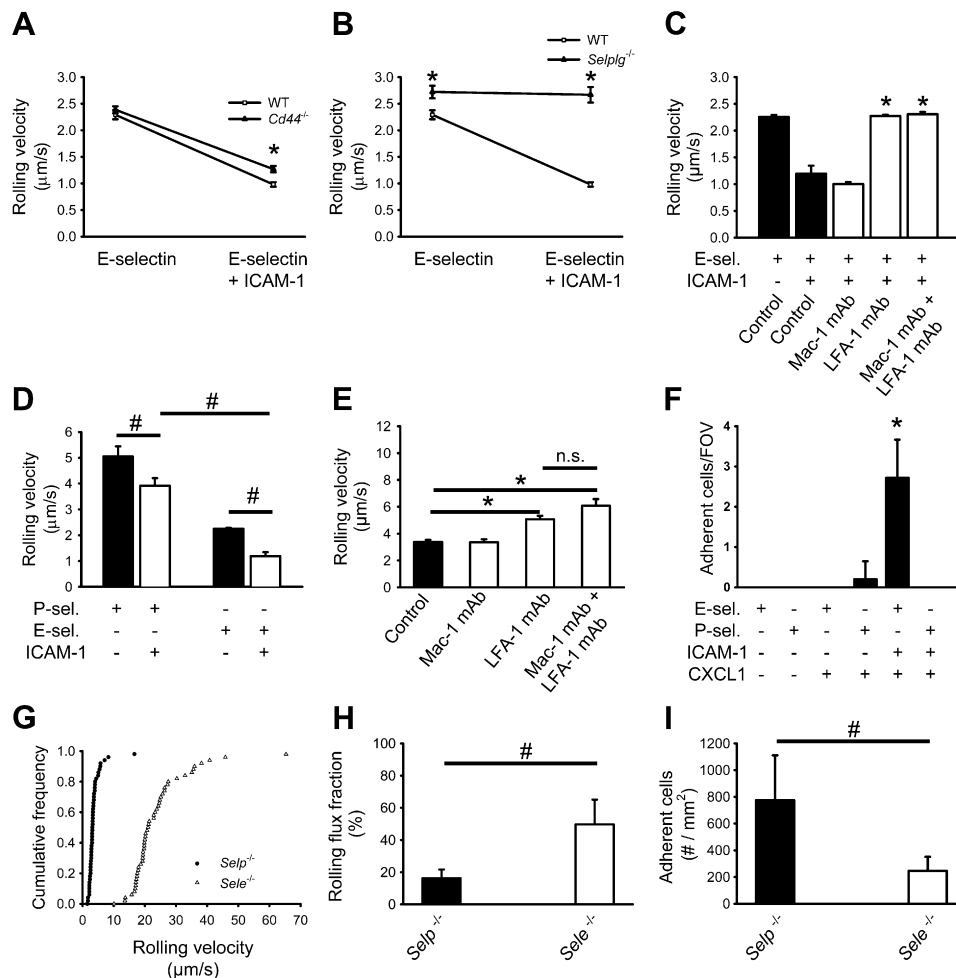
## RESULTS

### PSGL Engagement Triggers Slow Rolling

Because neutrophils are extremely sensitive to isolation procedures, all experiments were conducted in whole blood in vivo or in an autoperfused flow chamber at 5.94 dyn/cm<sup>2</sup> (Chesnutt et al., 2006; Smith et al., 2006). Some flow chambers were perfused with blood for 6 min, fixed, and stained for myeloperoxidase to identify neutrophils (data not shown). The rolling velocity of WT neutrophils was 2.2  $\mu$ m/s on E-selectin and decreased to 1.0  $\mu$ m/s on E-selectin and ICAM-1 (Figure 1A). To test the role of CD44 and PSGL-1 in E-selectin-dependent slow rolling, *Cd44*<sup>-/-</sup> or *Selp*<sup>plg</sup><sup>-/-</sup> mice were cannulated and connected to autoperfused flow chambers. *Selp*<sup>plg</sup><sup>-/-</sup> neutrophils in whole blood showed a slightly higher rolling velocity on E-selectin compared to WT neutrophils and a drastically elevated rolling velocity on E-selectin and ICAM-1 that was identical to the rolling velocity on E-selectin alone (Figure 1B). By contrast, *Cd44*<sup>-/-</sup> neutrophils rolled almost normally (Figure 1A). These results show that PSGL-1 on neutrophils is required for slowing down rolling neutrophils upon E-selectin engagement.

To examine which  $\beta_2$ -integrin is responsible for slow rolling on E-selectin and ICAM-1, we measured the rolling velocity of neutrophils of untreated mice before and after blocking of CD11a (LFA-1) and/or CD11b (Mac-1). In the autoperfused flow chamber, blocking CD11b had no effect on the rolling velocity compared to the control (Figure 1C). However, the inhibition of CD11a (LFA-1) led to an increase of rolling velocity to a level similar to that seen in the absence of ICAM-1, which was not further increased by blocking of both LFA-1 and Mac-1 (Figure 1C). Adding ICAM-1 to P-selectin-coated flow chambers also reduced rolling velocity by a similar amount as on E-selectin (Figure 1D), which is also largely LFA-1 dependent (data not shown).

To investigate which  $\beta_2$ -integrin is responsible for isolated E-selectin-induced slow rolling in vivo, we used intravital microscopy of cremaster muscle venules in P-selectin-deficient mice. 2 hr after TNF- $\alpha$  injection, the hemodynamic parameters were similar in all groups (Table 1), and average rolling velocity was 3.3  $\pm$  0.7  $\mu$ m/s (Figure 1E). Blocking of CD11b did not elevate the rolling velocity, whereas blocking of CD11a or both CD11a and



**Figure 1. PSGL-1, but Not CD44, Is Required for Neutrophil Slow Rolling on ICAM-1 upon E-Selectin Engagement**

(A–D) Carotid cannulas were placed in untreated mice and connected to E-selectin-coated and E-selectin-coated and ICAM-1-coated autoperfused flow chambers.

(A) Rolling velocity of neutrophils from WT and *Cd44*<sup>-/-</sup> mice presented as mean  $\pm$  SEM.

(B) Rolling velocity of neutrophils from untreated WT and PSGL-1-deficient mice on E-selectin and E-selectin+ICAM-1 presented as mean  $\pm$  SEM.

(C) Rolling velocity of untreated neutrophils on E-selectin+ICAM-1 before and after blocking LFA-1 or Mac-1 or both.

(D) Rolling velocity of neutrophils from untreated mice on P-selectin or E-selectin with or without ICAM-1.

(E) In vivo analysis of the rolling velocity of neutrophils in inflamed cremaster venules of P-selectin-deficient *Selp*<sup>-/-</sup> mice 2 hr after TNF- $\alpha$  injection.

(F) Neutrophil adhesion in the flow chamber after coimmobilization of CXCL1 (10  $\mu\text{g/ml}$ ) with P-selectin+ICAM-1 or E-selectin and ICAM-1. The wall shear stress in all flow chamber experiments was 5.94 dynes/cm<sup>2</sup>. At least three mice and four flow chambers per group.

(G–I) Intravital microscopy of cremaster muscle venules from P-selectin-deficient (*Selp*<sup>-/-</sup>) mice and E-selectin-deficient (*Sele*<sup>-/-</sup>) mice 2 hr after TNF- $\alpha$  application. Rolling velocity (G), rolling flux fraction (H), and number of adherent cells (I). At least 4 mice and 20 vessels per group.

\* $p < 0.05$ ; # $p < 0.05$ . Data presented are mean  $\pm$  SEM.

CD11b led to a significant increase ( $p < 0.05$ ). These data demonstrate that E-selectin-induced slow rolling is dependent on LFA-1 and not on Mac-1 in vitro and in vivo.

In order to investigate the physiological role of slow rolling on E-selectin and ICAM-1 compared to faster rolling on P-selectin and ICAM-1 (Figure 1D), we performed flow-chamber experiments with coimmobilized CXCL1 and measured neutrophil adhesion. CXCL1 at 10  $\mu\text{g/ml}$  induced neutrophil arrest on E-selectin and ICAM-1 but not on P-selectin and ICAM-1 in flow chambers perfused by untreated mice (Figure 1F). To confirm these findings

in vivo, we conducted intravital microscopy of the cremaster muscle of P-selectin-deficient mice and E-selectin-deficient mice 2 hr after TNF- $\alpha$  application and measured rolling velocities (Figure 1G), rolling flux fraction (Figure 1H), and number of adherent cells (Figure 1I). E-selectin-deficient mice showed a higher rolling velocity (Figure 1G), increased rolling flux fraction (Figure 1H), and reduced number of adherent cells (Figure 1I), compared to P-selectin-deficient mice. These in vivo and in vitro data show that slower rolling velocities induced by E-selectin and ICAM-1 support adhesion.

**Table 1. Diameter, Centerline Velocity, and Wall Shear Rate**

Antibody Treatment	Venules	Diameter ( $\mu\text{m}$ )	Centerline Velocity (mm/s)	Wall Shear Rate ( $1000 \text{ s}^{-1}$ )
Without Ab	18	$28 \pm 2$	$3.2 \pm 0.1$	$2.0 \pm 0.2$
M1/70	15	$27 \pm 2$	$3.1 \pm 0.1$	$2.0 \pm 0.1$
TIB-217	14	$26 \pm 2$	$3.1 \pm 0.1$	$2.0 \pm 0.2$
M1/70 + TIB-217	16	$29 \pm 2$	$3.1 \pm 0.1$	$1.9 \pm 0.1$

Diameter, centerline velocity, and wall shear rate are presented as mean  $\pm$  SEM of all investigated venules.

### Continuous E-Selectin Engagement Is Required to Sustain LFA-1-ICAM-1 Interactions

We previously showed that neutrophils did not roll in auto-perfused flow chambers coated with ICAM-1 alone (Chesnutt et al., 2006; Smith et al., 2006). To determine whether E-selectin engagement “switches on”  $\beta_2$ -integrin activation or whether continuous engagement is required to keep LFA-1 in an activated conformation, we coated one half of the chambers with E-selectin and ICAM-1 and the other half with ICAM-1. The cells rolled on E-selectin and ICAM-1 and immediately detached when they reached the ICAM-1-only zone (Figure 2A). Neutrophils rolling on E-selectin and ICAM-1 for 6 min immediately detached after injecting a monoclonal E-selectin antibody (9A9) (Figure 2B). These data show that continuous E-selectin-induced signaling is required to keep LFA-1 in an activated conformation; there is no apparent “memory” for prior engagement of E-selectin ligands.

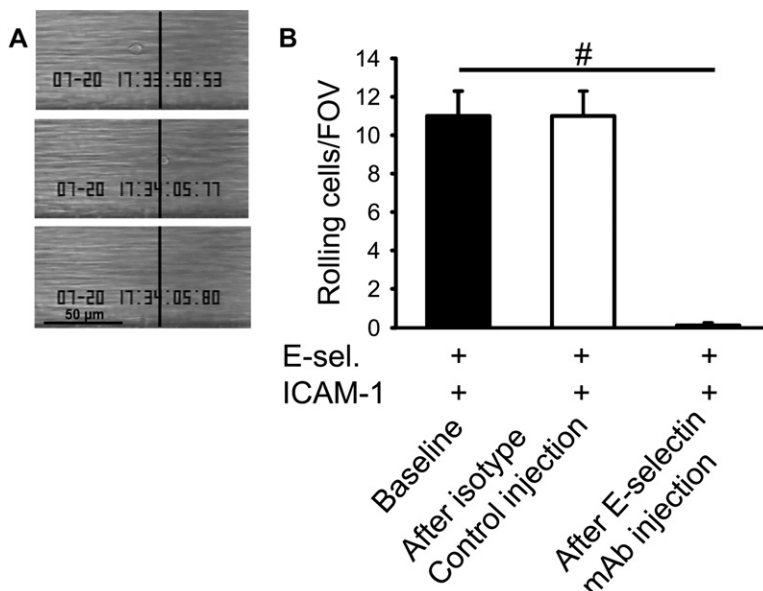
### Syk Is Required for Integrin Activation after E-Selectin and P-Selectin Binding to PSGL-1

The tyrosine kinase Syk is associated with the cytoplasmic tail of PSGL-1 and is also involved in downstream signaling (Urzainqui et al., 2002). Pretreatment of mice with piceatannol, an inhibitor of Syk, did not influence the rolling velocity of neutrophils on E-selectin or P-selectin,

but the decrease of rolling velocity on E-selectin and ICAM-1 was almost completely abolished after Syk blockade (Figure 3A and data not shown). To confirm our findings, chimeric mice were used that lack Syk expression in hematopoietic cells.  $\text{Syk}^{-/-}$  neutrophils showed a similar rolling velocity on E-selectin as WT neutrophils but failed to reduce their rolling velocity on E-selectin and ICAM-1 (Figure 3B). Similarly, blocking mitogen-activated protein (MAP)-kinase p38 did not influence the rolling velocity on E-selectin (data not shown) but partially elevated the rolling velocity on E-selectin and ICAM-1 (Figure 3C).

### TNF- $\alpha$ , but Not IL-1 $\beta$ , Induces Neutrophil Adhesion under Flow

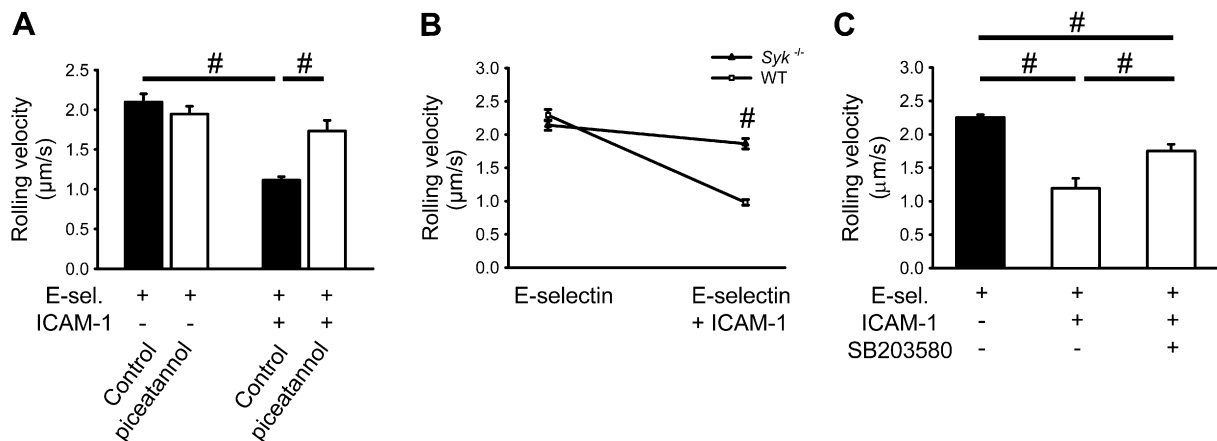
In many in vivo models, inflammation is induced by injecting TNF- $\alpha$ , which causes upregulation of adhesion molecules on endothelial cells, slow rolling, adhesion, and recruitment of leukocytes (Kunkel et al., 2000). Pretreatment of WT mice with TNF- $\alpha$  did not change the rolling velocity in auto-perfused flow chambers coated with E-selectin. After TNF- $\alpha$ , but not IL-1 $\beta$ , the rolling velocity on E-selectin and ICAM-1 dropped further (Figure 4A). TNF- $\alpha$ , but not interleukin-1 $\beta$  (IL-1 $\beta$ ), treatment also induced neutrophil adhesion on E-selectin and ICAM-1 (Figure 4B). Adhesion was induced by TNF- $\alpha$ -treated,



**Figure 2. Continuous E-Selectin Engagement Is Required to Keep LFA-1 in an Activated Conformation**

(A) The first half of auto-perfused flow chambers was coated with E-selectin+ICAM-1 and the second half with ICAM-1 alone, border indicated by vertical line. Cells rolled on E-selectin+ICAM-1 but detached immediately upon reaching ICAM-1 alone (representative of four independent experiments).

(B) Number of rolling neutrophils on E-selectin+ICAM-1-coated chambers before and after injection of a blocking E-selectin antibody (9A9). Data presented are the mean  $\pm$  SEM from at least four mice and four flow chambers ( $n = 4$ ).  $\#p < 0.05$ .



**Figure 3. Syk Is Involved in Integrin Activation after E-Selectin or P-Selectin Binding to PSGL-1**

Whole blood was perfused through E-selectin- and E-selectin+ICAM-1-coated autoperfused flow chambers at 5.94 dyn/cm<sup>2</sup>.

(A and B) The rolling velocity of neutrophils from mice pretreated with piceatannol (1 mg/mouse, [A]), a specific Syk inhibitor, and Syk<sup>-/-</sup> chimeric mice (B) were determined after 6 min (n = 4).

(C) Rolling velocity on E-selectin and E-selectin+ICAM-1 was measured after blocking of the mitogen-activated protein (MAP)-kinase p38 by the inhibitor SB203580 (100 μg/mouse i.p.) 1 hr prior to the experiment (n = 3).

#p < 0.05. Data presented are mean ± SEM.

but not IL-1β-treated, blood, showing that factors produced by blood cells are sufficient for this effect (Figure 4C). To test whether chemokine deposition on the surface of E-selectin- and ICAM-1-coated flow chambers during the perfusion of whole blood through the chamber was relevant, we perfused E-selectin- and ICAM-1-coated flow chambers with plasma from untreated or TNF-α-treated mice for 6 min. Then, these same flow chambers were connected to untreated mice. Perfusion of plasma from TNF-α-treated mice did not induce neutrophil adhesion (Figure S1 in the Supplemental Data available online).

In order to investigate which conformation of LFA-1 is responsible for slow rolling and adhesion, we used an allosteric inhibitor of LFA-1 (Compound 6845). This inhibitor is a structural analog of the allosteric inhibitor XVA143 (Shimaoka et al., 2003a), which has been shown to target the I-like domain on the β<sub>2</sub>-subunit and prevent firm adhesion by stabilizing the α<sub>4</sub>β<sub>2</sub> heterodimer, activating the β<sub>2</sub> I-like domain, and inhibiting activation of the α I domain. This inhibitor therefore stabilizes the intermediate affinity conformation of LFA-1. Pretreatment of mice with the allosteric inhibitor before TNF-α application did not influence the rolling velocity on E-selectin and E-selectin and ICAM-1 compared to vehicle-treated mice (Figure 4D), but abolished leukocyte adhesion in response to TNF-α (Figure 4E). These data show that intermediate-affinity LFA-1 is responsible for slow rolling, whereas the fully activated conformation with high affinity of LFA-1 is necessary for leukocyte adhesion to ICAM-1.

#### CXCR2, but Not Syk, Controls Neutrophil Arrest in Response to TNF-α

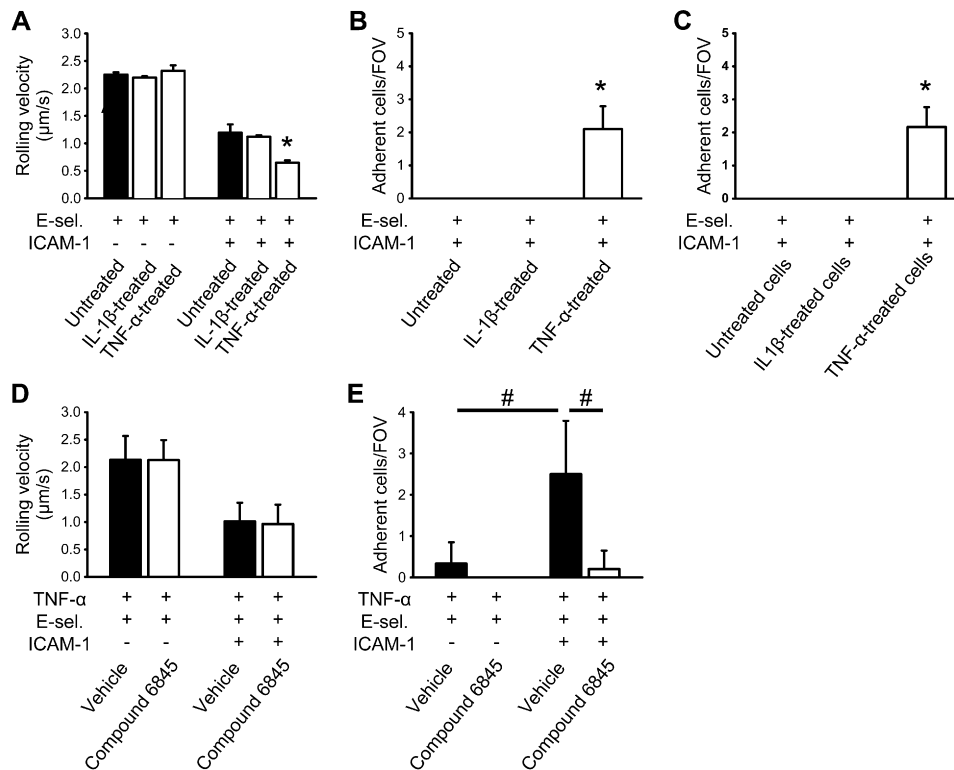
CXCR2 is an important chemokine receptor for neutrophil adhesion (Smith et al., 2004a). In order to investigate

whether GPCRs and specifically CXCR2 is involved in the modulation of rolling velocities and to address whether Syk is downstream of CXCR2, we used PTx to inhibit Gα<sub>i</sub>, piceatannol to inhibit Syk, and Syk<sup>-/-</sup> chimeric mice. PTx and piceatannol did not influence the rolling velocity on E-selectin (Figure 5A). Syk inhibition by piceatannol completely prevented the drop of rolling velocity on E-selectin and ICAM-1, whereas blocking of Gα<sub>i</sub> increased the rolling velocity by only 28% (Figure 5A). In contrast, PTx totally abolished neutrophil adhesion, whereas inhibition of Syk by piceatannol had no effect (Figure 5B).

In order to identify the GPCR involved in neutrophil adhesion, we used a blocking CXCR2 antibody. Blocking CXCR2 alone or in combination with Syk did not alter the rolling velocity on E-selectin. On E-selectin and ICAM-1, blocking CXCR2 had no effect on rolling velocity, but inhibition of Syk by piceatannol elevated the rolling velocity to the level seen on E-selectin (Figure 5C). TNF-α-induced adhesion was completely blocked by the CXCR2 antibody (Figure 5D). These findings were confirmed by intravital microscopy of mouse cremaster venules (Figure S2). Taken together, these results demonstrate that Syk is not involved in CXCR2-dependent arrest.

To investigate the mechanism by which TNF-α triggered CXCR2-dependent neutrophil arrest, we investigated CXCL1 production in neutrophils after TNF-α application in vivo. We stained the cremaster muscle for CXCL1 (Figures 5E and 5F) and measured the CXCL1 concentration in the plasma in the presence or absence of TNF-α (Figure 5G). Intrascrotal injection of TNF-α 2 hr before exteriorization induced an increase of adherent leukocytes and CXCL1 expression (Figure 5F) in venules of the cremaster muscle in WT mice compared to untreated mice (Figure 5E) and increased CXCL1 in plasma (Figure 5G). Taken together with the data presented above, we





**Figure 4. TNF- $\alpha$ , but Not IL-1 $\beta$ , Causes Neutrophil Arrest on E-Selectin + ICAM-1**

(A) Carotid cannulas of TNF- $\alpha$ - or IL-1 $\beta$ -pretreated mice were connected to E-selectin- and E-selectin+ICAM-1-coated autoperfused flow chambers. Rolling velocity of neutrophils was determined at a wall shear stress of 5.94 dyn/cm<sup>2</sup> (n = 4).

(B and C) Number of adherent neutrophils on E-selectin+ICAM-1 of TNF- $\alpha$ -pretreated mice (B) or TNF- $\alpha$ -pretreated whole blood (C) after 6 min.

(D and E) Mice were pretreated with TNF- $\alpha$  and an allosteric inhibitor (Compound 6845) of LFA-1 or vehicle, and rolling velocity (D) and number of adherent neutrophils (E) were determined on E-selectin and E-selectin+ICAM-1. To ensure that only LFA-1-dependent rolling and adhesion were investigated, all mice were treated with a blocking monoclonal antibody to Mac-1 (n = 4).

\*p < 0.05; #p < 0.05. Data presented are mean  $\pm$  SEM.

conclude that TNF- $\alpha$  induced CXCL1 activates LFA-1 through CXCR2 and G $\alpha_i$  to promote arrest, whereas ligation of PSGL-1 by E-selectin induces partial LFA-1 activation through Syk to promote slow rolling on E-selectin and ICAM-1 (Figure S3).

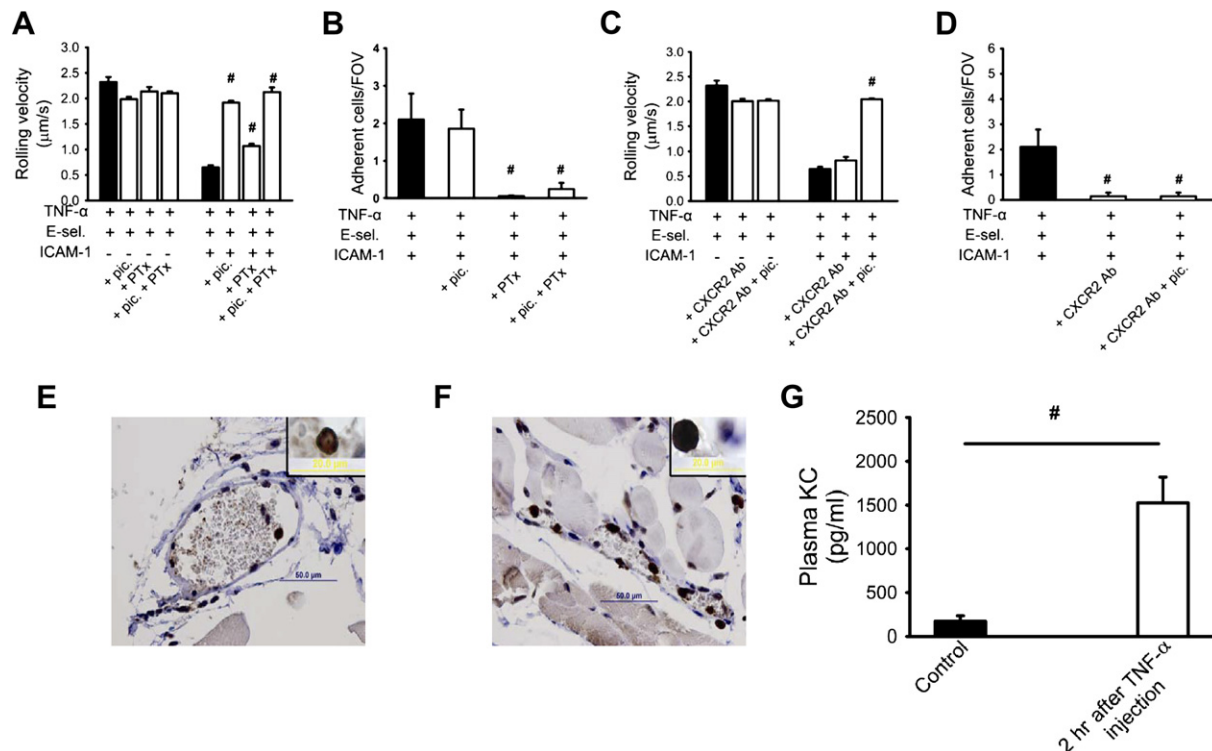
#### Inhibitions of Syk and GPCR Signaling Reduce Neutrophil Recruitment

The inability of neutrophils from PSGL-1-deficient mice to roll on ICAM-1 upon E-selectin engagement and the elimination of adhesion by blocking CXCR2 after TNF- $\alpha$  application suggests that inhibition of both PSGL-1 signaling and chemokine-mediated adhesion may prevent neutrophil influx into inflamed tissues. To compare the physiological importance of the two signaling pathways, neutrophil recruitment into thioglycollate-induced peritonitis was investigated in WT, *Selpg*<sup>-/-</sup>, and *Sele*<sup>-/-</sup> mice with or without PTx treatment. Consistent with previous data (Smith et al., 2004a), pretreatment with PTx reduced neutrophil recruitment to the peritoneum 4 hr after thioglycollate injection into WT mice (Figure 6). *Selpg*<sup>-/-</sup> mice also showed a reduced neutrophil influx after thioglycollate ap-

plication compared to WT mice (Yang et al., 1999). However, blocking both pathways by combining PTx treatment with the absence of PSGL-1 almost completely eliminated neutrophil recruitment into the peritoneal cavity after thioglycollate injection (Figure 6). In order to show Syk involvement, we used either *Syk*<sup>-/-</sup> bone-marrow chimeric mice or blocked Syk by the inhibitor piceatannol (Figure 6). Blocking of Syk by piceatannol showed no reduction of neutrophil influx (data not shown), but pharmacological blockade of G $\alpha_i$  in *Syk*<sup>-/-</sup> chimeric mice or blockade of both G $\alpha_i$  and Syk in WT mice almost completely abolished thioglycollate-induced neutrophil recruitment to the peritoneal cavity (Figure 6). These data suggest that PSGL-1-Syk signaling and chemokine-GPCR signaling together control neutrophil recruitment in this model of thioglycollate-induced peritonitis.

#### DISCUSSION

Many studies have demonstrated that E-selectin engagement leads to activation of neutrophils (Chesnutt et al., 2006; Green et al., 2004; Mattila et al., 2005; Simon



**Figure 5. CXCR2 and  $G\alpha_i$ , but Not Syk, Control Neutrophil Arrest in Response to  $TNF-\alpha$**

(A and B) Rolling velocity (A) and adhesion (B) of neutrophils from  $TNF-\alpha$ -pretreated mice on E-selectin- and E-selectin+ICAM-1-coated flow chambers after blocking  $G\alpha_i$  with PTx or Syk by piceatannol alone or in combination.

(C and D) Rolling velocity (C) and neutrophil adhesion (D) on E-selectin+ICAM-1-coated flow chambers after blockade of CXCR2 by antibody or of Syk by piceatannol alone or in combination after  $TNF-\alpha$  application.

(E and F) Cremaster muscles of WT mice were stained for CXCL1 without (E) and with (F)  $TNF-\alpha$ .  $TNF-\alpha$  injection induced an increase in adherent leukocytes in the microcirculation of the cremaster muscle in WT mice compared to untreated mice. Inserts show typical neutrophils stained for CXCL1 (E, F).

(G) CXCL1 concentration in plasma before and 2 hr after  $TNF-\alpha$  injection ( $n = 4$ ).

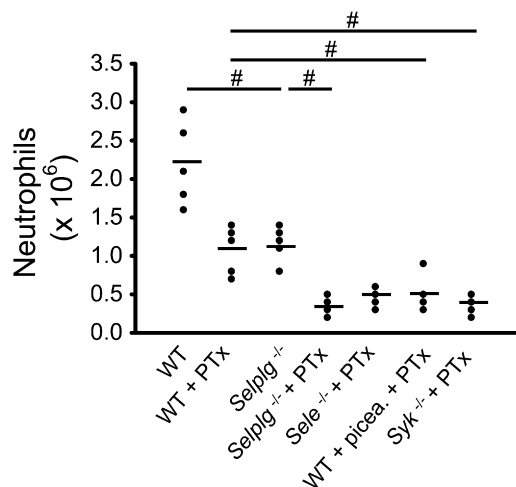
# $p < 0.05$ . Data presented are mean  $\pm$  SEM.

et al., 2000), but the ligand responsible for signaling was unknown. Here, we demonstrate that PSGL-1 is the signaling molecule responsible for LFA-1-dependent rolling upon E- or P-selectin engagement. This interaction induces Syk-dependent partial LFA-1 activation that causes slow rolling on ICAM-1, but not arrest. In response to  $TNF-\alpha$ , neutrophils produce CXCL1, which fully activates LFA-1 through CXCR2 and  $G\alpha_i$  in an autocrine or paracrine manner and causes firm adhesion.

Syk has previously been shown to be responsible for signaling events (Berton et al., 2005) secondary to outside-in signaling through integrins (Mocsai et al., 2002; Schymeinsky et al., 2006). Syk was needed for respiratory burst, degranulation, and spreading in response to proinflammatory stimuli (Mocsai et al., 2002) but not for G protein-coupled receptor signaling (Mocsai et al., 2003). Although Syk was previously shown to be activated by PSGL-1 (Urzainqui et al., 2002), our experiments with neutrophils in their native whole blood suggest that Syk is involved in the signaling pathway leading to  $\beta_2$ -integrin conformational change upon PSGL-1 binding to E-selectin or P-selectin. Syk-deficient neutrophils or Syk block-

ade increased rolling velocities on E-selectin and ICAM-1 similar to rolling velocities seen on E-selectin alone. We confirm that Syk is not involved in the signaling downstream of CXCR2 (Mocsai et al., 2003). Inhibition of Syk did not reduce the number of adherent neutrophils upon  $TNF-\alpha$  stimulation, and Syk chimeric mice showed a similar increase in leukocyte arrest upon CXCL1 application. Also, blocking Syk had no effect on rolling on E-selectin, suggesting that Syk blockade does not introduce gross morphological changes such as flattening of microvilli.

It has long been known that E-selectin induces slow rolling (Kunkel and Ley, 1996; Ley et al., 1998; Norman et al., 2000). This slow rolling also requires CD18 integrins (Dunne et al., 2002, 2003; Jung et al., 1998; Kunkel et al., 2000), but the mechanism of integrin activation was not understood. Our in vivo and in vitro data show that the  $\beta_2$ -integrin LFA-1, and not Mac-1, is responsible for E-selectin-induced slow rolling. Depending on the state of activation, integrins can be found in different conformations with different affinities to their ligands (Shimaoka et al., 2003b). The  $\beta_2$ -integrin LFA-1 in the intermediate-affinity conformation mediates rolling on ICAM-1, whereas



**Figure 6. Inhibition of  $G\alpha_i$  in *Selp1g*<sup>-/-</sup> Mice Blocks Neutrophil Recruitment In Vivo**

Peritoneal neutrophil influx 4 hr after 1 ml injection of 4% thioglycollate into WT mice, WT mice that had received 4  $\mu$ g PTx before thioglycollate injection, *Selp1g*<sup>-/-</sup> mice, *Selp1g*<sup>-/-</sup> mice plus PTx, *Sele*<sup>-/-</sup> mice plus PTx, WT mice that had received 1 mg piceatannol (picea) and 4  $\mu$ g PTx, and *Syk*<sup>-/-</sup> chimeric mice (*Syk*<sup>tm1Tyb</sup>) plus 4  $\mu$ g PTx ( $n = 5$  each). Data expressed as total numbers of neutrophils in the peritoneal lavage fluid counted by means of Kimura-stained samples, # $p < 0.05$ .

LFA-1 in the high-affinity and extended conformation induces arrest (Salas et al., 2004; Shimaoka et al., 2003b). An allosteric LFA-1 inhibitor that stabilizes the intermediate conformation did not influence slow rolling, suggesting that PSGL-1 binding to E-selectin induces the intermediate-affinity conformation of LFA-1. An alternative interpretation of our data is that some fraction of LFA-1 exists in the partially open, intermediate-affinity state, and that Syk is involved in outside-in signaling after ligand engagement, which may stabilize this conformation. By contrast, the chemokine-induced adhesion after TNF- $\alpha$  application was completely blocked by this allosteric inhibitor, suggesting that firm adhesion requires the high-affinity conformation of LFA-1.

The discovery of partial LFA-1 activation by PSGL-1 engagement was made possible by using neutrophils in whole blood rather than isolated neutrophils. Isolated neutrophils express increased levels of Mac-1 and show a decreased ability to roll (Forsyth and Levinsky, 1990; Glasser and Fiederlein, 1990; Kuijpers et al., 1991). As a case in point, isolated human neutrophils rolled at similar velocities on L-cells transfected with E-selectin alone or in combination with ICAM-1 (Simon et al., 2000), whereas we show a clear difference in rolling velocity on E-selectin versus E-selectin and ICAM-1. PSGL-1 engagement by E-selectin leads to slow rolling by inducing the partially activated conformation of LFA-1. This was probably missed in earlier studies, because isolated neutrophils are already partially activated.

TNF- $\alpha$  primes neutrophils and induces increased surface expression of CD11b, oxidative burst (Nathan et al., 1989; Richter et al., 1995), and chemokine production (Ha-

chicha et al., 1995). Here we show that TNF- $\alpha$  also triggers neutrophil adhesion on E-selectin and ICAM-1 through CXCR2 and  $G\alpha_i$ . Blocking of  $G\alpha_i$ -coupled receptors by PTx abolished neutrophil adhesion and also influenced rolling velocity. Antibody blockade of CXCR2 prevented neutrophil adhesion but did not influence the rolling velocity. These data demonstrate that CXCR2 is an important receptor for adhesion and suggest that other  $G\alpha_i$ -coupled receptors may be involved in controlling rolling velocity.

After TNF- $\alpha$  application, CXCL1 production in neutrophils and systemic CXCL1 concentrations increased. The treatment of isolated blood with TNF- $\alpha$  demonstrates that CXCL1 produced in neutrophils (Hachicha et al., 1995) activates CXCR2 in an autocrine or paracrine way and effectively induces arrest. Taken together, our results show that different signaling pathways for slow LFA-1-dependent rolling and arrest exist, but this study does not address where the  $G\alpha_i$ - and Syk-signaling pathways converge. Downstream molecules that have been implicated in integrin activation include p38 MAPK (Simon et al., 2000), ERM proteins (Urzainqui et al., 2002), and talin (Tadokoro et al., 2003).

Previous work has shown reduced neutrophil recruitment to thioglycollate-induced peritonitis in *Selp1g*<sup>-/-</sup> mice (Yang et al., 1999) and in PTx-treated mice (Smith et al., 2004a). Treating *Sele*<sup>-/-</sup> mice with PTx almost completely abolished neutrophil recruitment in this model (Smith et al., 2004a). Here, we directly show the relevance of the PSGL-1 pathway by demonstrating that PTx treatment of *Selp1g*<sup>-/-</sup> mice blocks neutrophil recruitment as effectively as PTx treatment of *Sele*<sup>-/-</sup> mice. These data show that both the PSGL-1-Syk pathway and the CXCR2- $G\alpha_i$  pathway are physiologically relevant in vivo. Therefore, therapeutic anti-inflammatory strategies targeting neutrophils must consider both chemokine and E-selectin pathways. Because most lymphocytes bind E-selectin poorly (Ley and Kansas, 2004), novel anti-inflammatory strategies may be able to spare lymphocyte trafficking and thus avoid unwanted immunosuppression.

## EXPERIMENTAL PROCEDURES

### Animals and Generation of Bone-Marrow Chimeras

We used 8- to 12-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), P-selectin, E-selectin, CD44, and PSGL-1-deficient mice backcrossed to C57BL/6 for at least 10 generations. Mice carrying the *Syk*<sup>tm1Tyb</sup> mutation (Turner et al., 1995) were maintained as *Syk*<sup>+/-</sup> heterozygotes on the C57BL/6 genetic background. The generation of mice with a *Syk*<sup>-/-</sup> hematopoietic system was performed as described previously (Mocsai et al., 2002). Mice were housed in a barrier facility under specific pathogen-free conditions. The Animal Care and Use Committee of the University of Virginia (Charlottesville) approved all animal experiments.

### Antibodies, Cytokines, Recombinant Proteins, and Other Reagents

Recombinant murine E-selectin-Fc, P-selectin-Fc, ICAM-1-Fc, tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and the blocking CXCR2 mAb (clone 242216) were obtained from R&D Systems (Minneapolis, MN). CXCL1 (keratinocyte-derived chemokine [KC]) was purchased from PeproTech (Rocky Hill, NJ). The Syk-inhibitor piceatannol



was purchased from A.G. Scientific (San Diego, CA) and was used in a concentration of 1 mg per mouse. The blocking mAb 9A9 (rat immunoglobulin G1 [IgG<sub>1</sub>], 30  $\mu$ g per mouse) against murine E-selectin was provided by B. Wolitzky (MitoKor, San Diego, CA). The blocking lymphocyte function antigen (LFA)-1 mAb TIB-217 (rat IgG<sub>2a</sub>, 30  $\mu$ g per mouse) was purified at the biomolecular facility of the University of Virginia from hybridoma supernatant (ATCC, Rockville, MD). The blocking Mac-1 mAb M1/70 (rat IgG<sub>2b</sub>, 30  $\mu$ g per mouse) was purchased from Pharmingen (San Diego, CA). The small molecule antagonist, Compound 6845, binds to the  $\beta_2$  subunit I-like domain and was a gift from G. Ju (Roche, Nutley, NJ). If not stated otherwise, all other reagents were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).

#### Surgical Preparation and Intravital Microscopy

Mice were anesthetized with an i.p. injection of ketamine hydrochloride (125 mg/kg, Sanofi Winthrop Pharmaceuticals, New York, NY), atropine sulfate (0.025 mg/kg, Fujisawa USA, Inc., Deerfield, IL), and xylazine (12.5 mg/kg, Tranquil Vet, Phoenix Scientific, Inc., St. Joseph, MO) and placed on a heating pad to maintain the body temperature at 37°C. After tracheal intubation (Polyethylene PE 90 tubing, ID 0.86 mm, OD 1.27 mm), cannulation of the carotid artery (PE 10, ID 0.28 mm, OD 0.61 mm) and flushing the cannula with 10 units heparin sodium per ml saline, the cremaster muscle was prepared for intravital microscopy as previously described (Ley et al., 1995). Microscopic observations were conducted on postcapillary venules ranging from 20 to 40  $\mu$ m in diameter with an intravital microscope (Axioskop; Zeiss, Thornwood, NY) with a saline immersion objective (SW 40, 0.75 numerical aperture). A CCD camera (model VE-1000CD, Dage-MTI) was used for recording. Surface area,  $S$ , was calculated for each vessel by  $S = \pi \cdot d \cdot l_v$ , where  $d$  is the diameter and  $l_v$  is the length of the vessel. Rolling flux was measured as the number of cells that roll past a line perpendicular to the vessel axis per minute. Leukocyte rolling flux fraction is defined as the flux of rolling leukocytes in percent of total leukocyte flux (Ley and Gaetgens, 1991). Leukocyte arrest was determined before and 1 min after intravenous injection of 600 ng CXCL1 as described previously (Smith et al., 2004a). Arrest was defined as leukocyte adhesion longer than 30 s and expressed as cells per surface area. Blood-flow centerline velocity was measured with dual photodiode and a digital online cross-correlation program (Circusoft Instrumentation, Hockessin, DE). Centerline velocities were converted to mean blood-flow velocities by multiplying with an empirical factor of 0.625 (Lipowsky and Zweifach, 1978).

#### Blood-Perfused Microflow Chamber

In order to investigate the rolling velocity, the number of rolling cells, and adherent cells, we used a recently described microflow chamber system (Chesnutt et al., 2006; Smith et al., 2006). 20  $\times$  200  $\mu$ m rectangular glass capillaries were filled with E-selectin (30  $\mu$ g/ml) or P-selectin (20  $\mu$ g/ml) alone or in combination with ICAM-1 (15  $\mu$ g/ml) and/or CXCL1 (10  $\mu$ g/ml) for 2 hr and blocked for 1 hr with 10% casein (Pierce Chemicals, Dallas, TX). The chamber was connected at one side to a PE 10 tubing and inserted into the carotid artery. The other side of the chamber was connected to a PE 50 tubing and used to control the wall shear stress, which was calculated as described (Smith et al., 2004b). Microscopy was conducted with a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 20, N.A. 0.5). Images were recorded with a 3CCD color video camera (model DXC-390, SONY Corporation, Japan) connected to a Panasonic S-VHS recorder. The chamber was perfused with blood for 6 min before one representative field of view was recorded for 1 min.

In some experiments, blood was collected by cardiac puncture and stimulated with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (10 ng/ml) for 30 min according to a previously published protocol (Thompson et al., 2001).

#### Chemokines

CXCL1 (keratinocyte-derived chemokine [KC]) concentration in plasma was measured in triplicates by enzyme-linked immunosorbent

assay kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) under baseline conditions and 2 hr after TNF- $\alpha$  injection. Paraffin-embedded cremaster sections (5  $\mu$ m) were incubated with a goat anti-mouse polyclonal antibody against KC (C-19, Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by a biotinylated secondary antibody (Vector Laboratories, CA) and avidin-biotin-peroxidase (Vector Laboratories). Sections stained without primary antibody served as negative controls.

#### Peritonitis Model

Peritoneal recruitment of leukocytes was induced as previously described (Smith et al., 2004a). In brief, 1 ml of sterile water containing 4% thioglycollate was injected i.p. 4 hr before the peritoneal lavage with 10 ml PBS (containing 2 mM EDTA) was performed. Total number of neutrophils was determined by Kimura staining. Some mice received tail-vein injections of 4  $\mu$ g PTx 2 hr before thioglycollate injection.

#### Statistics

Statistical analysis was performed with SPSS (version 14.0, Chicago, IL) and included one-way analysis of variance, Student-Newman-Keuls test, and t test where appropriate. All data are presented as mean  $\pm$  SEM.  $p < 0.05$  were considered significant.

#### Supplemental Data

Three figures are available at <http://www.immunity.com/cgi/content/full/26/6/773/DC1/>.

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#### Note Added in Proof

While this manuscript was in press, Frenette and colleagues reported on E-selectin ligands in neutrophils (Hidalgo, A., Peired, A.J., Wild, M.K., Vestweber, D., and Frenette, P.S. (2007). Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. *Immunity* 26, 477–489).